Intensive exercise does not preferentially mobilise skin-homing T cells and NK cells.

Running head: CLA+ lymphocytes and exercise

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Abstract

Purpose: This study investigated whether natural killer (NK) cells and CD8+ T cells expressing cutaneous lymphocyte antigen (CLA) – a homing molecule for endothelial cell leukocyte adhesion molecule 1 (ELAM-1), which enables transmigration to the skin – are selectively mobilised in response to acute exercise. ***Methods***: Nine healthy males (mean ± SD age: 22.1 ± 3.4 years) completed two exercise sessions: high-intensity continuous cycling ('continuous exercise' at 80% $\dot{V}O_{2\,MAX}$ for 20 min) and low-volume high-intensity interval exercise ('HIIE' at 90% $\dot{V}O_{2\,MAX}$ 10 × 1 min repetitions with 1 min recovery intervals). Blood was collected before, immediately- and 30 min post-exercise for cryo-preservation of peripheral blood mononuclear cells. CLA+ and CLA− cells were quantified within NK subpopulations ($CD56^{bright}$ 'regulatory' and $CD56^{dim}$ 'cytotoxic' cells) as well as the following CD8+ T cell subpopulations: naive ('NA'; CD45RA+CCR7+), central memory ('CM'; CD45RA−CCR7+), effector-memory ('EM'; CD45RA−CCR7−) and CD45RA-expressing effector-memory cells ('EMRA'; CD45RA+CCR7−). ***Results:*** CLA+ NK cells and CD8+ memory T cells increased in response to both exercise bouts, but, overall, their numerical contribution to the exercise lymphocytosis was inferior to CLA− cells, which increased to a much greater extent during exercise. Tellingly, the most exercise-responsive cells – effector memory CD8+ cells and $CD56^{dim}$ cells – were CLA−. ***Conclusions:*** A small subset of CLA+ lymphocytes are mobilised into blood during acute intensive exercise, but CLA+ cells are not major contributors to exercise lymphocytosis, thus providing preliminary evidence that the skin is not a major origin, or homing-destination, of exercise-sensitive lymphocytes.

Key words: T cells, inflammation, skin, homing

INTRODUCTION

Memory CD8+ T cells and natural killer (NK) cells are the largest contributors to the acute and transient lymphocytosis that occurs in peripheral blood during intensive exercise (6, 20, 36). Largely driven by their relative high density cell surface expression of β2-adrenergic receptors that induce detachment from the vascular endothelium upon stimulation by catecholamines, the mobilisation of NK cell and CD8+ T cell subsets is considered to be an evolved mechanism that facilitates effector cell recruitment to sites of potential or ongoing injury (10). Built on findings from animal models and using an analogy of immune cells as 'soldiers', it is proposed that naïve and central memory CD8+ T cells – which are slightly increased during exercise – traffic from the boulevards (i.e., 'bloodstream') back to the 'barracks' (i.e., the spleen, lymph nodes). On the other hand, tissue-specific memory CD8+ T cells – which mobilise to a larger extent during exercise – home to specific peripheral tissues (e.g., lungs, gut) to conduct immune-surveillance against potential pathogenic challenge. Finally, it is hypothesised that effector CD8+ T cells – which are mobilised to the greatest extent during exercise – are redeployed to 'battlefield' sites of wound healing (e.g. in the skin) following acute exercise (9, 10). However, whether these highly cytotoxic effector NK and CD8+ T cells, which are selectively increased in an acute and transient manner by acute exercise, have the propensity to migrate to peripheral cutaneous sites remains unknown.

In support of this model, it has been demonstrated in murine models that exercise redeploys large numbers of T cells to the Peyer's patches, lungs and bone marrow, reinforcing the idea that T cells are mobilised to sites of potential antigen encounter (i.e., lungs, gut), as well as to serve other important functions such as the provision of additional stimuli for haematopoiesis in the bone marrow (20). However, the aforementioned study was unable to measure homing to cutaneous sites or areas of ongoing inflammation. It has been shown that T cells and NK cells do show increased infiltration into sites of experimental inflammation (i.e., subcutaneous implantation of a surgical sponge treated with the lymphocyte-specific chemokine lymphotactin) in mice 24 to 48 hours after acute psychological stress (40). However, it remains unclear whether this increased cell infiltration is driven by stress-induced effector cell redistribution, or other mechanisms occurring in the days after the acute stressor.

70 An approach that is commonly used to investigate lymphocyte homing propensity in humans is the
71 assessment of cell surface adhesion molecule expression on cells; this approach can be used to reveal the
72 probable trafficking patterns of cells mobilised into the bloodstream during exercise. For example, studies
73 have shown that there is a selective influx of CD8+ memory cells into the bloodstream that exhibit lower
74 levels of lymphoid homing markers such as CD62L and CCR7 (6), thus providing evidence that exercise
75 mobilises CD8+ T cells with a homing capacity for peripheral tissues. Further research has shown that these
76 cells mobilised by exercise express adhesion molecules such as CD11a (15, 22), CD11b (17, 18), VLA-4
77 (very late antigen-4) and LPAM-1 (lymphocyte Peyer's patch adhesion molecule-1) (15), which enable
78 migration to peripheral sites including the bone marrow (23), Peyer's patches (43) and lungs (38). However,
79 the aforementioned adhesion molecules cannot be used exclusively for identifying skin-homing potential and
80 it remains uncertain whether exercise-responsive CD8+ T cells and NK cells have a skin-homing phenotype.

81 Cutaneous lymphocyte antigen (CLA) expression on lymphocytes can be used to determine whether
82 lymphocytes preferentially mobilised during intensive exercise exhibit a homing phenotype for cutaneous
83 sites. CLA is a specialised form of P-selectin glycoprotein ligand-1 (PSGL-1; CD162), a surface glycoprotein
84 expressed constitutively on all human peripheral-blood T cells. After post-translational modification, PSGL-1
85 bears a Sialyl-Lewis^X (sLe^X) moiety, termed CLA, which avidly binds CD62E (E-selectin), an adhesion
86 molecule which initiates the transmigration cascade to the skin, and which is also upregulated during
87 cutaneous inflammation (4, 27, 28, 32). Thus, most T cells in both normal and diseased cutaneous sites are
88 CLA+ (7, 29, 30). In support, leukocyte infiltration to inflammatory sites can be largely inhibited by a CLA
89 modifier (11). Taken together, analyses of CLA+ cell mobilisation can be used as proxy marker to reveal
90 whether cells mobilised by exercise have the phenotypic capacity to migrate to 'battlefield' sites in the skin
91 (10).

92 The primary objective of this study was to investigate whether CD8+ T cells preferentially mobilised
93 during intensive exercise stress exhibit a homing phenotype for cutaneous sites. To fulfil this objective, we
94 investigated the number of CLA-positive cells among CD8+ T cell subsets, to establish the contribution of
95 CLA-positive cells to the stepwise CD8+ lymphocytosis pattern previously observed in response to exercise
96 (6). We also extended the analyses of CLA+ and CLA− cells to NK cell populations – the largest responders

97 to exercise stress (6). The second objective of this study was to compare the magnitude of CD8+ T cell and
98 NK cell mobilisation in response to continuous high-intensity exercise (continuous exercise) and high-
99 intensity intermittent exercise (HIIE). HIIE is often referred to as High Intensity Interval Training (HIIT)
100 when repeated frequently over several weeks or months. This form of exercise has received considerable
101 attention as a short-duration and low-volume means of achieving similar health benefits to continuous
102 exercise. Given the increasing health benefits that have been observed with HIIE, and although we have
103 established in a prior report that CD8+ T cell and NK cell subset mobilisation is intensity dependent (6), the
104 effects of HIIE on changes to these cell subsets remains unknown.

METHODS

Participants

Ten healthy males were recruited to take part in this study as previously described (41, 42). Peripheral blood mononuclear cells (PBMCs) were available from nine of the ten participants, and were isolated from blood samples collected before, immediately after, and 30 min after two different forms of exercise, described below. All nine participants (age: 22.1 ± 3.4 years; height: 180.5 ± 6.1 cm; weight: 78.1 ± 11.0 kg; body mass index: 24.0 ± 3.1 kg.m^2; $\dot{V}O_{2\,MAX}$: 43.8 ± 4.1 ml.kg.min^{-1}) included in this study were non-smokers, and refrained from taking vitamin supplements and anti-inflammatory medication for fourteen days, and did not exercise and consume alcohol or caffeine for two days prior to experimental trials. All participants provided written informed consent and the study was approved by the Science, Technology, Engineering and Mathematics Ethical Review Committee at University of Birmingham (reference: ERN_12-0830).

Preliminary measurements

Height and weight were assessed using standard methods and cardiorespiratory fitness ($\dot{V}O_{2\,MAX}$) was measured during an incremental exercise test on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport*, Groningen, *Netherlands*). Following a three-minute warm up at 30 watts, workload was increased by 30 watts every minute, until volitional exhaustion. A facemask was fitted throughout preliminary exercise tests in each main trial, and expired air measured breath-by-breath averaged every 20 seconds for oxygen uptake and carbon dioxide production (*Oxycon Pro*, Jaeger, Wuerzberg, Germany). Heart rate (HR) was monitored every minute using a Polar Vantage heart rate monitor (Polar Vantage, Kempele, Finland); HR_{peak} represents the maximum heart rate achieved during each trial. The following criteria were used to indicate that $\dot{V}O_{2\,MAX}$ had been reached: a fall in cadence below 60 rpm, a respiratory exchange ratio ($\dot{V}CO_2$ / $\dot{V}O_2$) >1.10-1.15, a plateau in oxygen consumption and a heart rate >220 beats min^{-1} minus age.

Experimental trials

Experimental trials were undertaken at least seven days after preliminary measurements, in the morning, and following an overnight fast. Each trial was separated by at least three days in a randomised

132 design. Prior to each trial, but after the baseline blood sample, participants undertook a warm up (5 minutes) at
133 a workload eliciting 40% $\dot{V}O_{2\,MAX}$. Exercise trials were either vigorous steady state cycling at 80% $\dot{V}O_{2\,MAX}$
134 for 20 minutes ('continuous exercise') or high intensity interval exercise ('HIIE') trial. HIIE consisted of ten 1
135 minute cycling phases at a workload to elicit 90% $\dot{V}O_{2\,MAX}$, with 1 minute of low intensity cycling at 40%
136 $\dot{V}O_{2\,MAX}$ between each phase. Workload was expressed as watts, and relative to body mass (i.e., watts/kg).
137 Values presented are the average workload over the entire exercise protocol (i.e., in the vigorous trial: the
138 duration of cycling at 80% $\dot{V}O_{2\,MAX}$; and in the HIIE protocol: each 1-minute sprint phase of cycling at 90%
139 $\dot{V}O_{2\,MAX}$). Values were obtained directly from the electromagnetically braked cycle ergometer (Lode
140 Excalibur Sport, Groningen, Netherlands). Energy expenditure (Kcal) was estimated by indirect calorimetry
141 from calculations of fat and carbohydrate oxidation (g/min) and data were averaged over the 1-minute phases
142 of the protocols and summed to provide total energy expenditure from the entire duration of each trial,
143 expressed relative to body mass (Kcal/kg). Ratings of perceived exertion (RPE) were recorded using the Borg
144 Scale every 1 minute during continuous exercise, and after each 90% $\dot{V}O_{2\,MAX}$ interval during HIIE; the
145 highest RPE score was selected as the final RPE result for each exercise trial (RPE_{peak}).
146
147 *Blood sampling*
148 An intravenous cannula (*Becton & Dickson, Oxford, UK*) was inserted into an antecubital vein and
149 blood samples were drawn into potassium ethylene diaminetetraacetic acid (EDTA) vacutainer tubes (*Becton*
150 *& Dickson, Oxford, UK*). The cannula was kept patent with saline (0.9% NaCl). The leukocyte differential
151 was assessed using an automated haematology analyser (*Coulter Analyser, Beckman-Coulter, High Wycombe,*
152 *UK*).
153
154 *Blood Cell Isolation*
155 Approximately 15 ml of blood from each time point (pre-exercise, post-exercise and 30 min post-
156 exercise) was diluted 1:1 with Roswell Park Memorial Institute Media (RPMI), and then layered on top of
157 Ficoll paque PLUS (GE Healthcare) (2 blood : 1 Ficoll), before centrifuging at 500 × g for 30 minutes at
158 21°C. PBMCs were aspirated and washed three times in RPMI by centrifuging at 400 × g for 5 minutes. The

cell pellet was re-suspended in 1-ml of freezing mixture (70% RPMI, 20% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO)) and frozen at −1°C / min using a freezing container (Nalgene "Mr Frosty" Thermoscientific). Cells were stored at −80°C and analysed within six months.

Flow cytometry

Samples were thawed rapidly at 37°C and washed twice in PBS containing 2% FCS and 2mM EDTA by centrifuging at 400 × g for 5 minutes. Cells were counted using a haemocytometer and approximately 300,000 PBMCs were added to tubes for incubation with fluorescently conjugated antibodies to identify specific lymphocyte populations using eight-colour flow cytometry (FACS-CANTO, Becton–Dickinson, San Jose, USA). The following monoclonal antibodies (mAbs) were used: anti-CD45RA-FITC clone # HI100, anti-CD197 (CCR7)-PE clone # 150503, anti-CD56-PE-Cy7 clone # B159, anti-CD8-APC clone # RPA-T8, anti-CD3-APC-Cy7 clone # SK7 (BD Pharmingen, San Diego, USA), anti-CLA-V450 clone # HECA-452 (BioLegend, San Diego, USA) and anti-CD16-V500 clone # 3G8 (BD Horizon, San Diego, USA). In addition, 7-aminoactinomycin D (7-AAD; PerCP channel; BD Pharmingen, San Diego, USA) was used to exclude necrotic and apoptotic cells. For validation purposes, fluorescence-minus-one (FMO) tubes were used on separate samples from three healthy donors to establish negative and positive gating strategies for CLA expression.

Flow cytometry data were analysed using FlowJo version 7.6.5 (FlowJo LLC, Oregon, USA). Briefly, lymphocytes were gated on the forward *versus* sideways scatter, and 7AAD− cells were divided into CD3+ or CD3− cells. Cytotoxic T cells were identified as being CD3+CD8+ and further differentiated into naïve ('NA'; CCR7+CD45RA+), central memory ('CM'; CCR7+CD45RA−), effector memory ('EM'; CCR7−CD45RA−) or CD45RA+ effector memory cells ('EMRA'; CCR7−CD45RA+). CD3− cells were further differentiated into $CD56^{dim}$ 'cytotoxic' NK cells or $CD56^{high}$ 'regulatory' NK cells, as proposed by Cooper and colleagues (2001), using CD16 and CD56 dotplot gating (8). Within each subpopulation identified above, cells were subdivided based on their expression of CLA (CLA+ or CLA−) in SSC (side scatter) versus V450-CLA mode. Absolute cell counts for each subpopulation were computed by multiplying the percentage of cells within the CD3+ or CD3− gates by the total lymphocyte count (obtained via the Coulter principle).

This value and subsequent absolute subpopulation counts were multiplied by percentage values for gated daughter subpopulations.

Statistical analyses

All results are presented as mean ± standard deviation unless otherwise stated. Statistical calculations were performed on IBM SPSS for Windows Version 21. Kolmogorov-Smirnov tests confirmed that all data were normally distributed at all time points and for all variables. Repeated Measures Analysis of Variance (ANOVA) tests were used to contrast changes over time (baseline, exercise and 30 min post-exercise), and between the two exercise conditions (HIIE and continuous exercise) for each cell type. Within-trial main effects of time for each cell type were calculated in separate individual ANOVAs. Post-hoc pairwise comparisons were made with Bonferroni adjustments for multiple comparisons. Between trial differences at a given time point were assessed by paired *t*-tests. Statistical significance was accepted at the $p < .05$ level.

198 **RESULTS**

199 All participants completed both the continuous exercise and HIIE tasks. Total energy expenditure was
200 significantly higher after continuous exercise compared to HIIE (HIIE: 2.69 ± 0.31 Kcal/kg; continuous
201 exercise: 3.54 ± 0.48 Kcal/kg; $F_{(1,17)} = 20.0$, $p < .05$). Similarly, RPE_{peak} was significantly higher after
202 continuous exercise compared to HIIE (HIIE: 16 ± 2 (Borg scale); continuous exercise: 18 ± 1 (Borg scale);
203 $F_{(1,17)} = 4.6$, $p < .05$). Workload was higher after HIIE compared to continuous exercise (HIIE: 2.74 ± 0.54
204 Watts/kg; continuous exercise: 2.18 ± 0.33 Watts/kg; $F_{(1,17)} = 7.1$, $p < .05$). No differences were found
205 between trials for HR_{peak} (HIIE: 178 ± 14 beats/min; continuous exercise: 187 ± 7 beats/min; $p > .05$).

206

207 *Effects of HIIE and continuous exercise on the number of total leukocytes and leukocyte subpopulations*

208 Total leukocyte counts increased ($p < .05$) from baseline after both the HIIE (5.31 ± 1.15 [$x10^9$/L] to
209 8.92 ± 2.77) and continuous exercise (5.57 ± 0.94 to 10.73 ± 2.14) conditions. Similarly, total lymphocyte
210 counts also increased ($p < .05$) from baseline after both the HIIE (1.83 ± 0.47 to 3.64 ± 1.46) and continuous
211 exercise (1.91 ± 0.41 to 4.83 ± 1.33) conditions, with a larger mean change observed during continuous
212 exercise, compared to HIIE, but these differences were not significant ($p > .05$). 30 min after each exercise
213 condition, lymphocyte numbers between trials were similar (HIIE: 1.67 ± 0.37; continuous exercise: 1.84 ±
214 0.35) and had returned to pre-exercise levels ($p > .05$, compared to baseline).

215

216 *Effects of HIIE and continuous exercise on the number of total CD3+ T cells and CD3+CD8+ T cells*

217 Table 1 displays total CD3+ T cells and CD3+CD8+ T cell numbers during both exercise tasks. Total
218 CD3+ T cells increased significantly during both conditions ($p < .01$), with larger mean increases during
219 continuous exercise, compared to HIIE, but these between trial differences were not significant ($p > .05$). 30
220 min after each condition, CD3+ T cell numbers between trials were similar and had returned to pre-exercise
221 levels. CD3+CD8+ T cells increased significantly during both conditions ($p < .05$), with larger increases
222 during continuous exercise compared to HIIE; again, these differences between trials were not significant ($p >$
223 .05). 30 min after each condition, CD3+CD8+ numbers between trials were similar and had returned to pre-

exercise levels. Replicating previous findings (6, 39), CD3+CD8+ T cell subsets were mobilised in a stepwise manner, with CD3+CD8+ EMRA T cells mobilising more than EM, CM and NA cells. Within these subsets, there were no significant differences in the magnitude of mobilisation between continuous exercise or HIIE trials.

Effects of HIIE and continuous exercise on the numbers of CLA+ and CLA− CD3+ T cells and CD3+CD8+ T cells

Approximately 14% of total CD3+ T cells were CLA+ at baseline, and despite an increase in the number of CLA+ CD3+ T cells during both exercise conditions ($p < .05$), the proportion of CLA+ CD3+ T cells decreased slightly during exercise due to a larger influx of CLA− CD3+ T cells ($p = .05$) (see Table 1). At 30 minutes post-exercise the number and proportion of CLA+ CD3+ T cells returned to pre-exercise values.

Approximately 9% of CD3+CD8+ T cells were CLA+ at baseline, and, in a similar manner to total CD3+ T cells, the number of CLA+ CD3+CD8+ T cells increased during both exercise conditions ($p < .05$), before returning to baseline levels at 30 minutes post-exercise (see Table 1). However, there was no change ($p > .05$) in the proportion of CLA+ CD3+CD8+ T cells during exercise. No differences were observed in the magnitude of mobilisation between CLA+ and CLA− cells in any of these broad T cell subsets between HIIE and continuous exercise.

Effects of HIIE and continuous exercise on the numbers of CLA+ and CLA− CD3+CD8+ T cell subsets

In accordance with prior literature (7, 31), very few of the CD3+CD8+ NA cell population expressed CLA (median = 1.35%) and the few CLA+ naïve cells did not change during exercise (data not shown). The largest proportion of CLA+ CD3+CD8+ T cells were of the CM (~20%) and EM (~23%) phenotypes, with only a small fraction of EMRA cells expressing CLA (<7%). As illustrated in Figure 1, the percentage change in the number of CLA+ cells increases in a stepwise mobilisation pattern (i.e., EMRA > EM > CM) in

response to both exercise conditions, and this pattern is also evident in CLA− cells. However, as illustrated in Figure 2, the proportion of CLA+ cells per memory CD3+CD8+ T cell subset actually shows a marginal and significant (p<0.05) decline during exercise, before returning to baseline levels at 30 min post-exercise. Thus, CLA+ cells do not appear to be major contributors to the large influx of effector cells observed in the peripheral bloodstream during exercise; this is illustrated by a greater mobilisation of CLA−, compared to CLA+, EMRA CD3+CD8+ T cells in Figure 1.

Effects of HIIE and continuous exercise on the numbers of NK cells, and CLA+ and CLA− cells within the NK cell subsets

Table 2 displays changes in the numbers of CD3−CD56+ NK cells in response to continuous exercise and HIIE. As expected, NK cells were extremely sensitive to exercise stress, exhibiting significant differences between the two exercise modalities (p < .05), with HIIE resulting in a ~400% increase and continuous exercise resulting in a ~600% change during exercise. With regards to the major CD56+ NK cell subsets, $CD56^{dim}$ 'cytotoxic' cells were the most sensitive to exercise stress, with HIIE resulting in a 550% increase and continuous exercise resulting in a 725% increase (p <.05). As expected, $CD56^{bright}$ 'regulatory' NK cells were less sensitive to exercise stress and no differences were found between exercise conditions (p >.05); HIIE resulted in a 100% increase and continuous exercise resulted in a 200% increase.

At baseline, approximately ~22% of CD3−CD56+ cells were CLA+, with a higher proportion of $CD56^{bright}$ 'regulatory' NK cells positive for CLA (~67%) than $CD56^{dim}$ 'cytotoxic' NK cells (~17.5%). During exercise, the total number of CLA+ NK cells increased (HIIE: ~230%; continuous exercise: ~350%), and, as expected, this was driven by a larger increase in CLA+ $CD56^{dim}$ cells compared to the CLA+ $CD56^{bright}$ cells. As a consequence of a greater influx of CLA− NK cells (HIIE: ~500%; continuous exercise: ~650% compared to baseline), primarily comprised of CLA−$CD56^{dim}$ cells during exercise (Figure 3), the proportion of CLA+ cells in the total NK cell pool was actually reduced during exercise (Figure 4). Thus, CLA+ NK cells do not appear to be major contributors to the large influx of effector cells observed in the peripheral bloodstream during exercise.

DISCUSSION

This study assessed the mobilisation propensity of CLA+ CD8+ T cells into the peripheral bloodstream in response to intensive exercise. A preferential mobilisation of this cell phenotype would fulfil a component of the stress redistribution theory (10), which hypothesises that effector memory cells preferentially mobilised by exercise have a phenotype that enables post-exercise migration to cutaneous sites of wound healing or active inflammation (e.g., injured skin). We examined the number of CLA+ and CLA− cells because CLA avidly binds CD62E; CD62E is thought to be the primary initiator of routine CD8+ T cell transmigration to the skin, and is also central to T cell tethering to cytokine-activated endothelium at inflamed cutaneous sites (14). We found that exercise resulted in an increase in the number of memory CLA+ CD3+CD8+ T cells. However, the numerical contribution of CLA+ memory cells to exercise-induced lymphocytosis was inferior to CLA− cells, and, as a consequence, the proportion of CLA+ cells amongst the total memory CD8+ T cell pool showed a decline during exercise. Furthermore, the most exercise-sensitive subset (EMRA CD8+ T cells) were largely CLA−, suggesting that the most exercise-responsive T cell subset mobilised by exercise do not have a phenotype that would enable rapid transmigration to sites of active cutaneous inflammation.

Until now, the effect of exercise on the mobilisation of CLA+ cells has not yet been investigated. A study investigating the effects of acute psychological stress on circulating CLA+ CD8+ cells found a marginal decline in the number of CLA+ CD8+ cells during the stressor (2). These aforementioned results differed to the findings of our study, as we observed an approximate doubling in the number of CLA+ CD3+CD8+ cells during exercise. It was proposed by Atanackovic et al. (2006) that CLA+ CD8+ T cells had already initiated transmigration to sites of inflammation during acute stress. However, evidence exists to suggest that elevated levels of epinephrine selectively decreases adhesion of CD8+ T cells (12) and NK cells (3) to endothelial cells, and may thus be a contributing mechanism for the maintenance of CLA+ cells in the peripheral bloodstream, as observed in our exercise trials. We extended our CLA+ analyses to four distinct CD8+ T cell subsets conventionally identified using the cell-surface markers CCR7 and CD45RA (17). In agreement with prior literature showing that the majority of CLA+ CD3+ cells are CD45RO+ (7), we found that few naïve (CCR7+CD45RA+) CD8+ T cells expressed CLA (31). This did not change in response to exercise. In further

agreement with Clark et al. (2006), we found a majority of CLA+ cells were of the central memory (CCR7+ CD45RA−) phenotype and fewer were effector memory (CCR7−CD45RA−) CD8+ T cells; these CLA+ effector memory cells showed the largest increase during exercise, though the numerical increase was small.

On one hand, the findings of this study are supportive of the stress redistribution model in that exercise evoked an increase in the total number of peripheral blood CLA+ CD8+ T cells, which are phenotypically consistent with skin-homing T cells. On the other hand, the absolute number of CLA+ cells mobilised was small, and the relative magnitude of mobilisation was much less than CLA− CD8+ cells. Furthermore, few EMRA CD8+ T cells – conventionally the most exercise-sensitive CD8+ subset – expressed CLA, and did not increase in number as much as CLA− EMRA CD8+ T cells. Thus, the CD8+ T cells with the greatest propensity for exercise-induced mobilisation and extravasation are CLA− and do not exhibit a phenotype characteristic for rapid homing to cutaneous sites or sites of endothelial inflammation. Given that the large majority of CLA+ cells reside in the skin (7), it is possible to conclude that the skin is not a major contributor/source of cells to exercise-induced lymphocytosis. The findings of this study are not surprising: CLA+ cells are not as susceptible to immunosenescence (25), and are thus strikingly different to the most exercise-sensitive cells, which have shorter telomeres, high expression of CD57 (35, 36); and exhibit exaggerated exercise-induced mobilisation in participants seropositive for cytomegalovirus (39). In addition, it has been demonstrated in rodents that exercise redeploys large numbers of T cells to the lungs (20). Lung-derived T cells are all CLA−, but positive for other homing molecules including CCR5 and CXCR3 (5). Similarly, exercise also redeploys T cells to the Peyer's patches and bone marrow (19, 20) and this is likely governed by other site-specific homing molecules like LPAM-1 (37) or VLA-4 (23, 26) rather than CLA. This allocation of certain cells to defined parts of the body might represent a homeostatic immune-surveillance response (10), or, instead, it has been hypothesised that senescent T cells are mobilised into the blood to facilitate their subsequent apoptosis in peripheral tissues (21, 34), which may contribute to progenitor cell mobilisation after exercise (24). With regards to cutaneous surveillance against tumours and stressed tissue cells, it is unlikely this is tasked by αβ CD8+ T cells alone (13), and is likely supported by γδ T cells and NK cells, which are highly responsive to acute stress (1, 6), and have the migratory capacity to enter cutaneous sites (13). We found that CLA+ CD56+ NK cells were substantially mobilised by exercise, but, much like CD8+ T cells, were outnumbered by the mobilisation of CLA− NK cells.

This is the first study to investigate and compare the effects of HIIE (also referred to as HIIT when repeated over several weeks or months) and continuous exercise on T cell and NK cell subset mobilisation responses. HIIE typically involves shorter and more intense bouts of exercise than more traditional forms (e.g., 30 minutes of moderate intensity running or cycling) and is thought to be more attractive and better tolerated by participants. HIIE has received considerable recent attention as an effective means of achieving certain physiological adaptations (e.g., improved insulin sensitivity and cardiorespiratory fitness, but probably not weight loss) in healthy and diseased populations that are similar or superior to traditional endurance-based exercise (16).

Although we found a trend whereby a greater mobilisation of lymphocytes was observed during continuous exercise compared to HIIE, these differences were not significant for the majority of lymphocyte subpopulations investigated. We found that CD56 NK cells – conventionally the most exercise responsive cells – were mobilised to a greater extent during continuous exercise compared to HIIE, a response that was driven by larger increases to $CD56^{dim}$ NK cells. It may be that similar intensity-dependent effects would have been found for CD8+ T cells, and other subsets, if a larger group of participants had been tested.

A limitation of this study was that post-exercise blood samples were collected 30 minutes after exercise cessation, rather than after 60 minutes, when NK and CD8+ T cell lymphocytopenia is more pronounced (6, 39). This was a consequence of practical and logistical constraints imposed by the broader study that was being undertaken (41). At 30 min post-exercise, we found that all cell phenotypes were present in peripheral blood in similar numbers to pre-exercise levels. It would be of interest to investigate the contribution of CLA+ T cells to lymphocytopenia at later time points. An additional practical limitation was the cryopreservation of PBMCs, which may disproportionately affect the viability of some cell phenotypes – however this is unlikely to affect the within-subject model used in our study. A further consideration for future studies surrounds the ongoing debate over the optimal phenotyping of skin-homing CD8+ T cells. In addition to CLA, CCR4 and CCR8 may be useful in differentiating between CD8+ T cells involved in normal cutaneous immune-surveillance to those involved in acute or chronic inflammation (7, 33). Finally, our investigations were limited to healthy individuals with no apparent cutaneous inflammation. Investigation of exercise-induced lymphocyte skin-homing in experimental models of infection or in clinical populations

including psoriasis is warranted, and more invasive techniques such as biopsy may provide further insights into exercise-induced homing to cutaneous or inflammatory sites.

CONCLUSION

A greater number of CLA− T cells and NK cells were mobilised into peripheral blood than CLA+ counterpart T cells and NK cells during exercise. Furthermore, the majority of EMRA T cells and $CD56^{dim}$ cells – i.e., conventionally the most exercise-responsive cells – did not express CLA. Together, these findings demonstrate that CLA+ cells are not major contributors to exercise lymphocytosis, thus providing preliminary evidence that the skin is not a major origin, or homing-destination, of exercise-sensitive lymphocytes. We conclude that the most exercise-sensitive lymphocytes likely migrate from, and to, non-cutaneous sites post-exercise.

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FIGURE CAPTIONS

Figure 1. Mean ± SEM changes in the proportion of CD8+ T cell subsets (CM = central memory; EM = effector memory; EMRA = CD45RA+ effector memory) that were either positive or negative for cell surface CLA, at baseline and immediately after Continuous exercise. It was found that CLA+ CD45RA+ effector memory CD8+ T cells were mobilised to a greater magnitude than CLA− CD45RA+ effector memory CD8+ T cells; similar patterns were found for HIIE (data not shown).
* Indicates significant difference between CLA+ and CLA− ($p < .05$).

Figure 2. Mean ± SEM changes in the proportion of CD8+ T cell subsets (NA = naïve; CM = central memory; EM = effector memory; EMRA = CD45RA+ effector memory) that were positive for CLA, from pre- to immediately post-continuous exercise. A reduction in the proportion of CLA+ CM, EM and EMRA cells was observed immediately after continuous exercise compared to baseline; similar patterns were observed during HIIE (data not shown).
* Indicates significant differences in the proportion of CLA+ cells between pre- and post-continuous exercise ($p < .05$).

Figure 3. Mean ± SEM changes in the proportion of NK cell subsets that were either CLA+ or CLA−, at baseline and immediately after continuous exercise. A trend was observed whereby CLA− $CD56^{dim}$ cells were mobilised to a greater extent than CLA+ $CD56^{dim}$ NK cells ($p = .058$); similar patterns were found for HIIE (data not shown).

Figure 4. Mean ± SEM changes in the proportion of NK cell subsets that were CLA+, from pre- to immediately post-continuous exercise. A reduction in the proportion of CLA+ $CD56^{bright}$ and $CD56^{dim}$ NK cells was observed from pre- to post-exercise; similar patterns were observed during HIIE (data not shown).
* Indicates significant differences in the proportion of CLA+ cells between pre- and post-continuous exercise ($p < .05$).

Table 1. Changes in the numbers of T-lymphocytes and T-lymphocyte subpopulations in response to HIIE and continuous exercise (mean ± SD).

Cells per µL	Condition	Baseline	Exercise	+30min	Main effects of time	Time × task interaction
CD3+	HIIE	1494.4 ± 406.2	2508.8 ± 1076.5*	1330.2 ± 293.2	$F_{(2,16)} = 16.18$; $p = .002$	$F_{(2,32)} = 2.06$; p = NS
	Continuous Ex	1454.1 ± 321.9	3049.6 ± 794.4***	1416.3 ± 363.1	$F_{(2,16)} = 42.97$; $p < .001$	
CD3+ CLA+	HIIE	189.2 ± 89.7	316.2 ± 237.0	186.8 ± 95.7	$F_{(2,16)} = 6.80$; $p = .030$	$F_{(2,32)} = .37$; p = NS
	Continuous Ex	183.0 ± 91.4	350.6 ± 166.6**	196.7 ± 123.2	$F_{(2,16)} = 26.06$; $p < .001$	
CD3+ CLA−	HIIE	1305.2 ± 376.2	2192.6 ± 894.2**	1143.4 ± 260.2	$F_{(2,16)} = 17.72$; $p = .002$	$F_{(2,32)} = 2.33$; p = NS
	Continuous Ex	1271.0 ± 293.3	2699.0 ± 276.0***	1219.6 ± 291.7	$F_{(2,16)} = 41.40$; $p < .001$	
CD3+ CD8+	HIIE	509.5 ± 181.9	1000.9 ± 654.7	463.1 ± 174.7	$F_{(2,16)} = 9.46$; $p = .014$	$F_{(2,32)} = .89$; p = NS
	Continuous Ex	491.2 ± 162.3	1185.0 ± 398.8***	490.4 ± 195.4	$F_{(2,16)} = 48.99$; $p < .001$	
CD3+ CD8+ CLA+	HIIE	41.3 ± 21.6	77.9 ± 65.6	43.2 ± 29.3	$F_{(2,16)} = 6.22$; $p = .035$	$F_{(2,32)} = .28$; p = NS
	Continuous Ex	40.7 ± 26.2	86.9 ± 53.8**	43.8 ± 32.7	$F_{(2,16)} = 23.95$; $p = .001$	
CD3+ CD8+ CLA−	HIIE	468.2 ± 163.4	923.0 ± 591.5	420.0 ± 148.7	$F_{(2,16)} = 9.76$; $p = .013$	$F_{(2,32)} = .95$; p = NS
	Continuous Ex	450.4 ± 141.2	1098.1 ± 355.0***	446.5 ± 166.9	$F_{(2,16)} = 48.96$; $p < .001$	
Naïve CD8+	HIIE	239.9 ± 93.1	341.6 ± 144.1*	192.8 ± 62.2	$F_{(2,16)} = 14.14$; $p < .001$	$F_{(2,32)} = 1.51$; p = NS
	Continuous Ex	215.9 ± 76.1	377.9 ± 69.0***	198.5 ± 56.4	$F_{(2,16)} = 55.18$; $p < .001$	
Naïve CD8+ CLA+	HIIE	3.4 ± 1.4	5.2 ± 2.6	3.0 ± 1.4	$F_{(2,16)} = 10.20$; $p = .001$	$F_{(2,32)} = .97$; p = NS
	Continuous Ex	3.5 ± 1.8	6.3 ± 3.4*	3.5 ± 2.2	$F_{(2,16)} = 17.67$; $p = .002$	
Naïve CD8+ CLA−	HIIE	236.5 ± 92.2	336.4 ± 142.3*	189.8 ± 62.0	$F_{(2,16)} = 14.13$; $p < .001$	$F_{(2,32)} = 1.49$; p = NS
	Continuous Ex	212.4 ± 75.8	370.5 ± 70.3***	195.0 ± 55.4	$F_{(2,16)} = 54.09$; $p < .001$	
CM	HIIE	118.8 ± 37.2	221.8 ± 97.1*	124.7 ± 55.9	$F_{(2,16)} = 21.50$; $p = .001$	$F_{(2,32)} = 3.85$; p = NS
	Continuous Ex	121.9 ± 64.2	278.6 ± 96.2***	132.3 ± 85.4	$F_{(2,16)} = 101.54$; $p < .001$	
CM CD8+ CLA+	HIIE	20.2 ± 8.3	35.6 ± 25.0	21.7 ± 12.7	$F_{(2,16)} = 7.01$; $p = .026$	$F_{(2,32)} = .29$; p = NS
	Continuous Ex	20.0 ± 11.8	38.9 ± 20.7**	21.3 ± 14.7	$F_{(2,16)} = 25.16$; $p < .001$	
CM CD8+ CLA−	HIIE	98.6 ± 30.4	186.2 ± 72.9**	103.1 ± 44.2	$F_{(2,16)} = 27.81$; $p < .001$	$F_{(2,32)} = 5.69$; $p = .017$
	Continuous Ex	101.9 ± 53.5	239.7 ± 77.4***	110.6 ± 71.8	$F_{(2,16)} = 123.76$; $p < .001$	
EM	HIIE	64.4 ± 29.9	144.9 ± 70.8**	69.5 ± 37.2	$F_{(2,16)} = 26.74$; $p = .001$	$F_{(2,32)} = 3.30$; p = NS
	Continuous Ex	66.6 ± 39.3	184.8 ± 71.0***	75.6 ± 52.0	$F_{(2,16)} = 80.73$; $p < .001$	
EM CD8+ CLA+	HIIE	12.3 ± 10.5	24.7 ± 27.5	13.7 ± 13.4	$F_{(2,16)} = 4.73$; p = NS	$F_{(2,32)} = .13$; p = NS
	Continuous Ex	12.0 ± 10.8	26.9 ± 24.5*	13.6 ± 14.0	$F_{(2,16)} = 11.22$; $p = .010$	
EM CD8+ CLA−	HIIE	52.1 ± 20.5	120.2 ± 44.7***	55.8 ± 24.6	$F_{(2,16)} = 40.39$; $p < .001$	$F_{(2,32)} = 5.08$; $p = .026$
	Continuous Ex	54.6 ± 29.7	157.8 ± 51.3***	62.2 ± 40.4	$F_{(2,16)} = 90.05$; $p < .001$	
EMRA	HIIE	88.3 ± 87.0	292.6 ± 390.3	76.1 ± 69.7	$F_{(2,16)} = 4.07$; p = NS	$F_{(2,32)} = .13$; p = NS
	Continuous Ex	86.6 ± 57.7	344.6 ± 296.6*	83.8 ± 52.1	$F_{(2,16)} = 9.16$; $p = .016$	
EMRA CD8+ CLA+	HIIE	5.4 ± 4.0	12.4 ± 12.6	4.9 ± 3.7	$F_{(2,16)} = 5.35$; $p = .047$	$F_{(2,32)} = .29$; p = NS
	Continuous Ex	5.4 ± 4.4	14.6 ± 10.9**	5.4 ± 4.6	$F_{(2,16)} = 17.13$; $p = .003$	
EMRA CD8+ CLA−	HIIE	80.9 ± 84.0	280.2 ± 378.7	71.2 ± 66.8	$F_{(2,16)} = 4.02$; p = NS	$F_{(2,32)} = .12$; p = NS
	Continuous Ex	81.4 ± 54.4	329.9 ± 291.0*	78.4 ± 48.6	$F_{(2,16)} = 8.75$; $p = .018$	

*$p < .05$ in comparison to baseline

** $p < .01$ in comparison to baseline

*** $p < .001$ in comparison to baseline

NS $p > .05$

Table 2. Changes in NK cell numbers in response to HIIE and continuous exercise (mean ± SD).

Cells per µL	Condition	Baseline	Exercise	+30min	Main effects of time	Time × task interaction
CD56+	HIIE	112.4 ± 44.8	591.9 ± 361.0**	115.2 ± 59.5	$F_{(2,16)} = 18.27$; $p = .003$	$F_{(2,32)} = 4.47$; $p = .05$
	Continuous Ex	164.3 ± 144.2	1130.6 ± 707.5**	157.6 ± 159.9	$F_{(2,16)} = 22.64$; $p = .001$	
CD56+CLA+	HIIE	28.2 ± 10.4	92.7 ± 47.8**	29.2 ± 12.4	$F_{(2,16)} = 23.96$; $p = .001$	$F_{(2,32)} = 3.37$; p = NS
	Continuous Ex	31.7 ± 9.5	142.1 ± 69.2**	31.9 ± 10.9	$F_{(2,16)} = 26.10$; $p = .001$	
CD56+CLA−	HIIE	84.1 ± 44.6	499.3 ± 352.6*	86.0 ± 56.5	$F_{(2,16)} = 14.13$; $p = .005$	$F_{(2,32)} = 3.75$; p = NS
	Continuous Ex	132.6 ± 136.6	988.4 ± 698.6**	125.7 ± 153.2	$F_{(2,16)} = 18.14$; $p = .003$	
$CD56^{bright}$	HIIE	11.0 ± 7.4	23.9 ± 19.4*	15.2 ± 13.0	$F_{(2,16)} = 9.98$; p =.011	$F_{(2,32)} = 2.17$; p = NS
	Continuous Ex	12.8 ± 7.0	36.0 ± 25.7*	15.6 ± 7.8	$F_{(2,16)} = 11.05$; $p = .009$	
$CD56^{bright}$ CLA+	HIIE	7.6 ± 5.5	15.0 ± 12.7*	10.2 ± 9.1	$F_{(2,16)} = 9.05$; p = .015	$F_{(2,32)} = 1.32$; p = NS
	Continuous Ex	8.4 ± 4.7	20.3 ± 15.8*	10.2 ± 5.7	$F_{(2,16)} = 8.88$ p = .015	
$CD56^{bright}$ CLA−	HIIE	3.4 ± 2.0	8.9 ± 7.2*	5.0 ± 4.0	$F_{(2,16)} = 10.14$; $p = .011$	$F_{(2,32)} = 1.79$; p = NS
	Continuous Ex	4.4 ± 2.7	15.8 ± 14.4	5.4 ± 2.5	$F_{(2,16)} = 6.35$; $p = .035$	
$CD56^{dim}$	HIIE	101.4 ± 44.5	568.0 ± 343.6**	99.9 ± 55.6	$F_{(2,16)} = 18.41$; p =.003	$F_{(2,32)} = 4.49$; $p = .05$
	Continuous Ex	151.5 ± 47.2	1094.5 ± 229.9**	141.9 ± 53.1	$F_{(2,16)} = 22.72$; $p = .001$	
$CD56^{dim}$ CLA+	HIIE	20.6 ± 10.0	77.7 ± 43.8**	19.0 ± 7.6	$F_{(2,16)} = 22.68$; p = .001	$F_{(2,32)} = 3.21$; p = NS
	Continuous Ex	23.3 ± 7.8	121.8 ± 60.8**	21.7 ± 8.9	$F_{(2,16)} = 25.51$; p = .001	
$CD56^{dim}$ CLA−	HIIE	80.7 ± 44.0	490.3 ± 345.8*	81.0 ± 55.0	$F_{(2,16)} = 14.16$; $p = .005$	$F_{(2,32)} = 3.75$; p = NS
	Continuous Ex	128.2 ± 135.3	972.7 ± 689.1**	120.3 ± 152.3	$F_{(2,16)} = 18.19$ $p = .003$	

*$p < .05$ in comparison to baseline

** $p < .01$ in comparison to baseline

NS $p > .05$

Figure 1

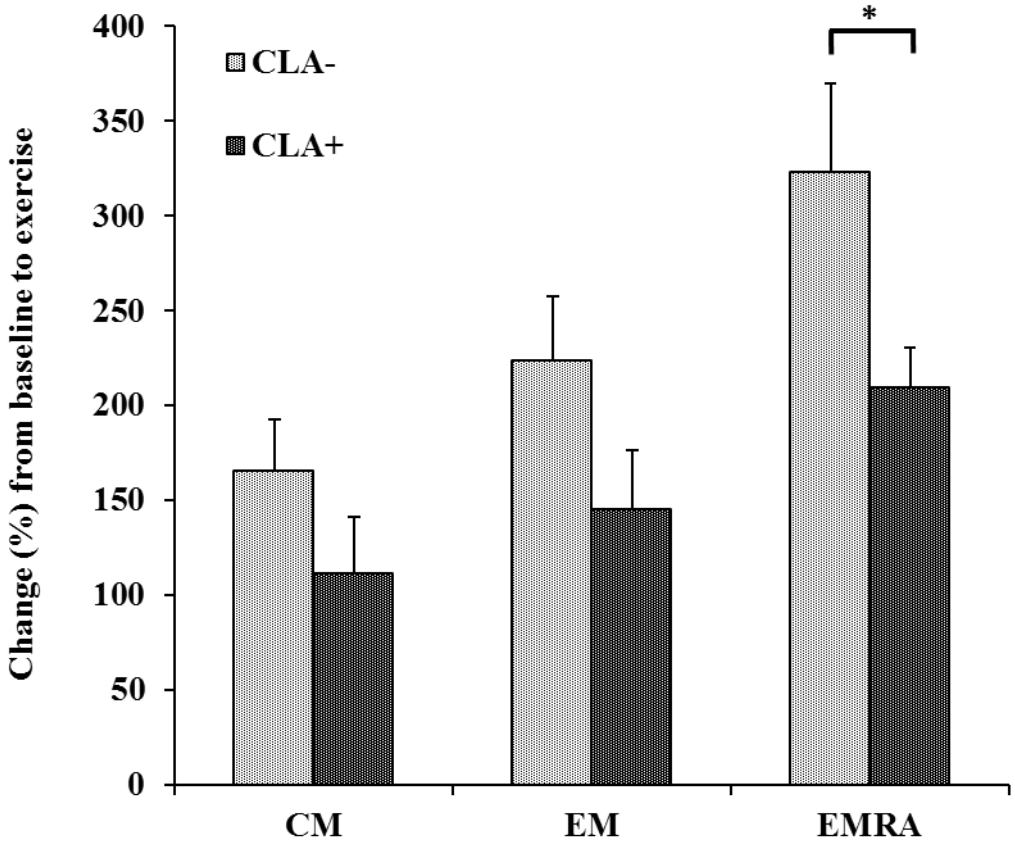
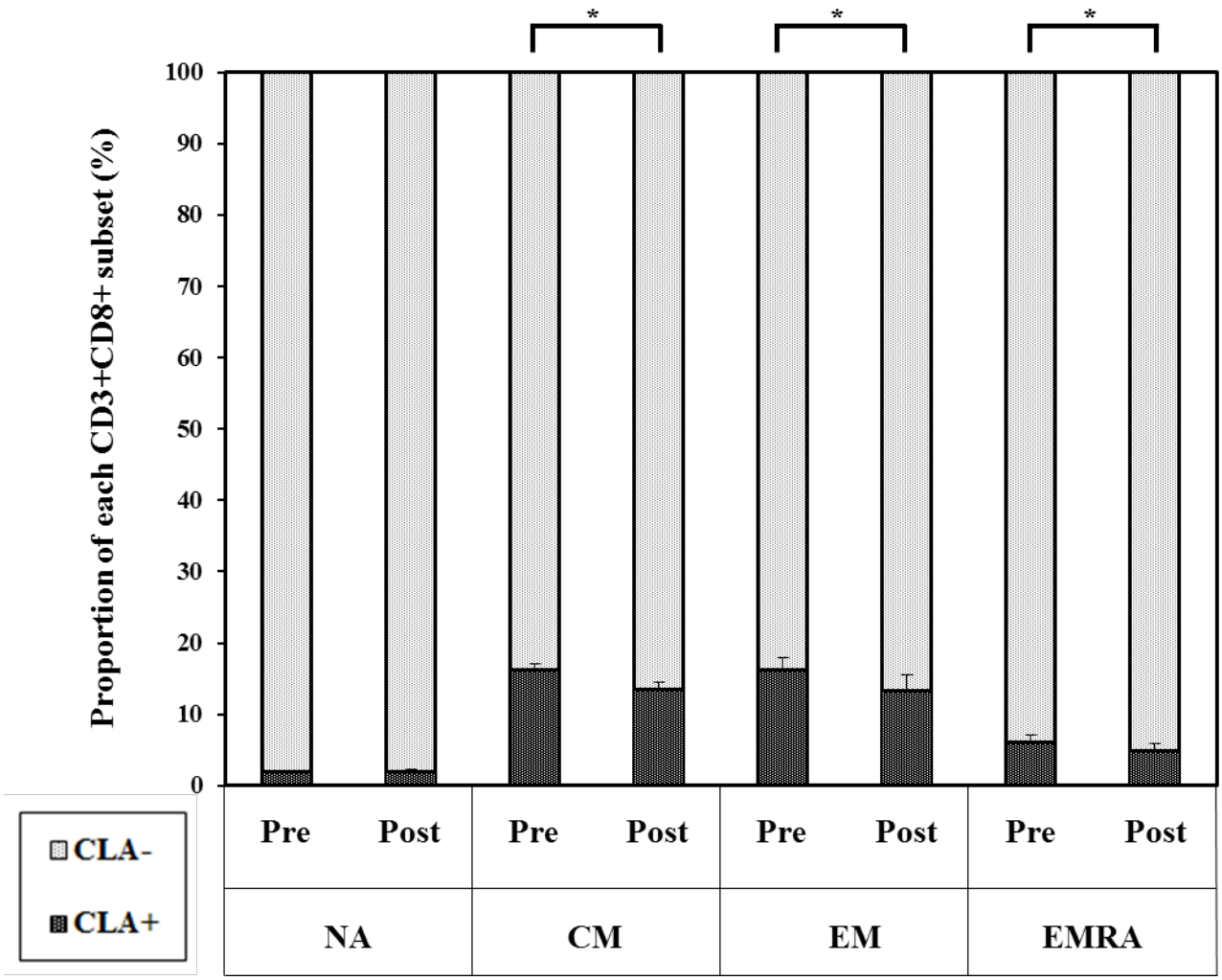

Change (%) from baseline to exercise
400
350
300
250
200
150
100
50
0
CLA-
CLA+
*
CM
EM
EMRA

Figure 2


*
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Proportion of each CD3+CD8+ subset (%)
100
90
80
70
60
50
40
30
20
10
0
CLA-
CLA+
Pre
Post
Pre
Post
Pre
Post
Pre
Post
NA
CM
EM
EMRA

Figure 3

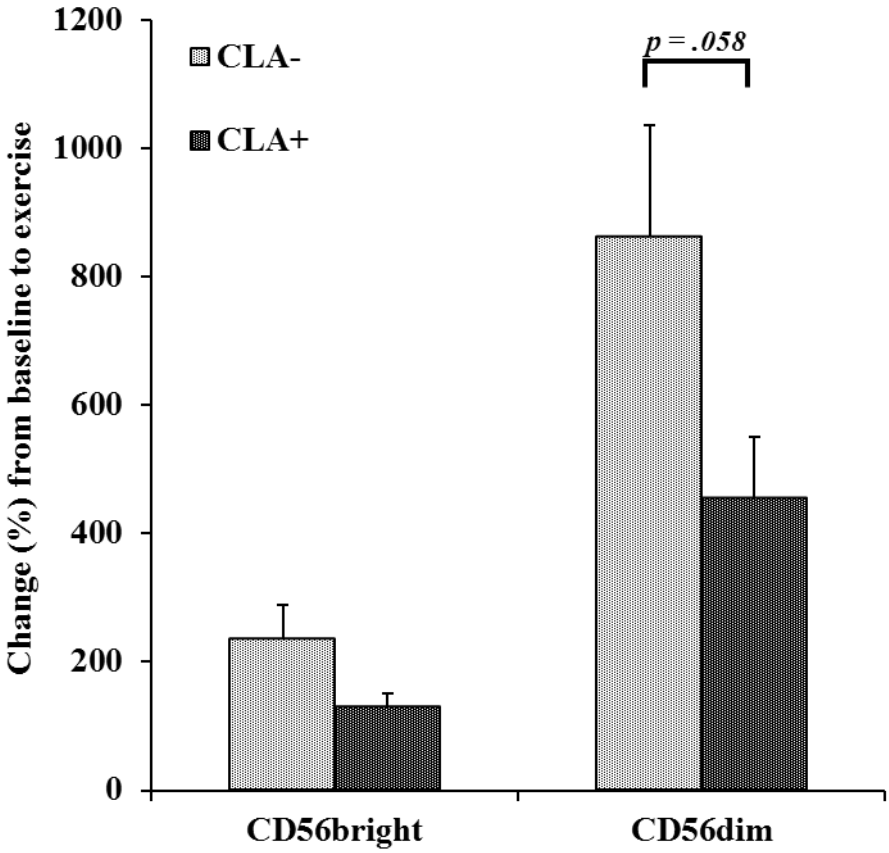
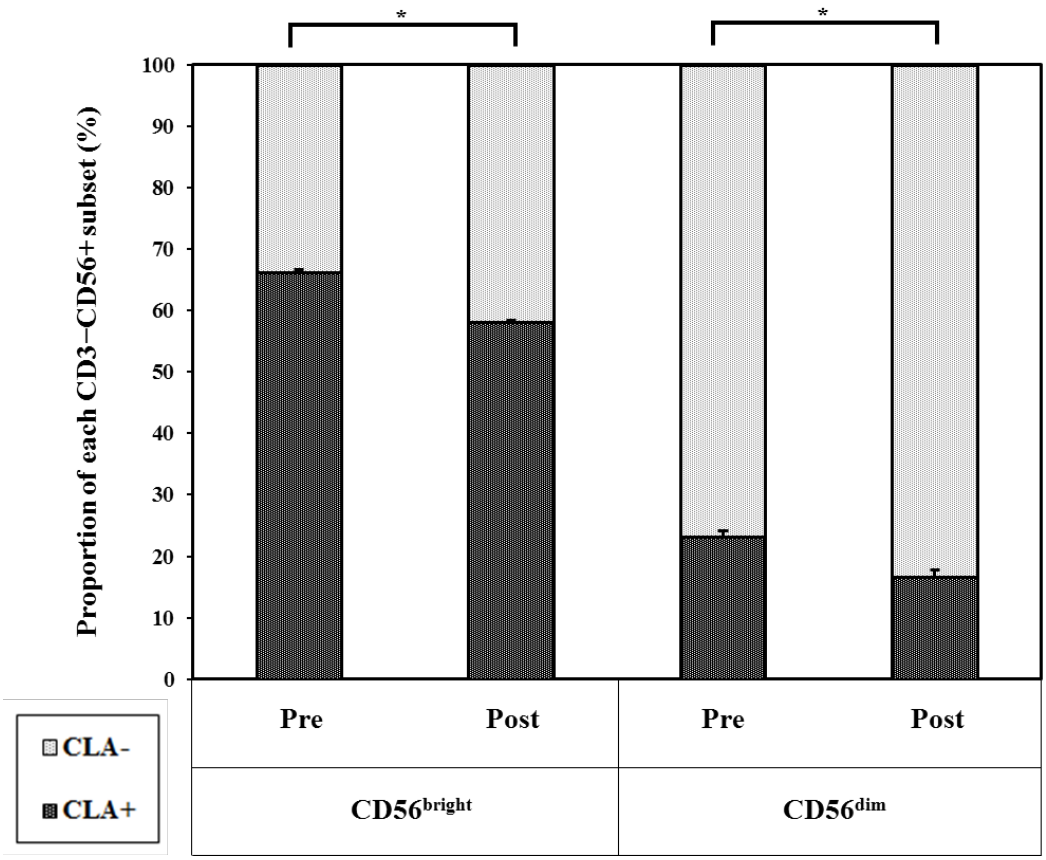

1200
1000
800
600
400
200
0
Change (%) from baseline to exercise
CLA-
CLA+
p = .058
CD56bright
CD56dim

Figure 4


*
*
100
90
80
70
60
50
40
30
20
10
0
Proportion of each CD3−CD56+ subset (%)
CLA-
CLA+
Pre
Post
Pre
Post
CD56bright
CD56dim